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GRANT NUMBER DAMD17-94-J-4103

TITLE: A Genetic Screen for Ligand Binding by the Human Estrogen Receptor

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REPORT DATE: September 1996

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

2

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*

2. REPORT DATE
September 1996

3. REPORT TYPE AND DATES COVERED
Annual (15 Aug 95 - 14 Aug 96)

4. TITLE AND SUBTITLE
A Genetic Screen for Ligand Binding by the Human Estrogen Receptor

5. FUNDING NUMBERS
DAMD17-94-J-4103

6. AUTHOR(S)
Dr. Mark D. Nichols

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
European Molecular Biology Organisation
Heidelberg, 69012, Germany

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, MD 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

19961216 037

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

FLP recombinase-steroid receptor fusion proteins convert ligand binding into DNA recombination. We describe a ligand responsive FLP - estrogen receptor binding domain (FLP-EBD) in yeast that accurately reflects known estrogen receptor agonist affinities. All tested estrogens, whether agonists or antagonists, induce FLP-$EBD_{251-595}$ recombination, indicating that all induce EBD release from the Hsp90 complex. Altering the distance between FLP and the EBD domains in the fusion protein affects ligand inducibility. A FLP-$EBD_{304-595}$, with 53 fewer amino acids, shows reduced inducibility by agonists, and unexpectedly, complete insensitivity to induction by all antagonists tested. Thus we observe a tethered interference between FLP and the EBD domains that differs depending on the distance between the two domains and the conformations induced by agonists or antagonists, presenting a distinction between estrogen agonists and antagonists in yeast. Combining this distinction with mutagenesis of the EBD has generated numerous mutations with altered ligand specificity, sometimes inverting the activation effects of hormones and antihormones. Further study will define the specific mechanisms leading to antihormone action, especially with respect to the therapeutically important antihormones, tamoxifen and raloxifene.

14. SUBJECT TERMS Breast Cancer, Tamoxifen, Hormone Binding Domain, in vitro Mutagenesis, Saccharomyces Cerevisieae, FLP Recombinase, DNA Recombination

15. NUMBER OF PAGES
36

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Mark D. Nichols 9-9-96

PI - Signature Date

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A Genetic Screen for Ligand Binding by the Human Estrogen Receptor.

Introduction

The molecular study of the steroid hormone receptors, including the estrogen, progesterone, glucocorticoid, androgen, and mineralocorticoid receptors, has determined that they display a modular structure composed of six domains, A to F (14). The two most conserved domains in the family are domains C and E, separated by a variable and nonconserved D domain sequence. Domain C is 66-68 amino acids long and has two zinc fingers, which mediate sequence-specific DNA binding. Domain E, also known as the ligand binding domain (LBD), is approximately 240 amino acids long (51) and mediates numerous overlapping functions: ligand binding, dimerization, Hsp90 binding, transrepression, transcriptional activation and modulation of other receptor functions, including cellular localization (4,25). Understanding of domain C is extensive, but the complexity of multiple functions in domain E (LBD) has impeded progress. Mutations within LBDs have revealed some insight into the structure/function relationship. The recent x-ray crystal structures of the related retinoid and thyroid nuclear receptor LBDs presents a conceptual framework for understanding how LBDs function (8,32,36,43,47). Nevertheless, many questions of LBD function remain unanswered. In particular, the properties that determine whether a ligand functions as a hormone (agonist) or an antihormone (antagonist) remains unclear.

Steroid hormone receptors pass through several stages during activation from their unliganded forms to fully active transcription factors. Binding of hormone causes a conformational change in the receptor which dissociates the Hsp90 complex (correlating with the size transition from 8-9S to 4-5S in glycerol gradients), allowing receptor dimerization (11,37). Dimers of the estrogen receptor, for example, are then competent to bind specific estrogen response elements (EREs) upstream of estrogen-regulated genes (5,19). Upon DNA binding, two domains of the receptor protein are capable of transcriptional activation functions (AFs): a hormone-independent AF-1 in the A/B domain and the hormone-dependent AF-2 within the E (LBD) domain. AF-1 and AF-2 functions vary in importance with individual promoters and cell-types and the net activity of AF-2 varies according to whether the bound ligand is an agonist or an antagonist (6,26,28,46).

The LBDs of the steroid receptors also have the ability to repress the function of certain proteins to which they are fused, thereby rendering their function dependent on ligand (33). This property probably results from the ability of LBDs to complex with Hsp90 (and partners) in the absence of ligand (35). Ligand binding releases the LBD from the Hsp90 complex, freeing the fusion protein to locate to its site of activity (38).

Previously it had been shown that fusing LBDs onto transcription factors and oncoproteins successfully conveyed repression and ligand dependency onto the activities of the fusion proteins. Recently we showed that LBD regulation could also be used to convey ligand dependency onto the activity of the site specific recombinase FLP (23) from the yeast 2 micron episome. FLP recognizes DNA binding elements, termed FLP recognition targets (FRTs), that consist of a 13-bp inverted repeat separated by an 8 bp spacer (15). Recombination occurs between two FRTs and the product of recombination is determined by the spacer orientation. Thus deletions, inversions, integrations or translocations are possible (42).

FLP recombinase-LBD fusion proteins, when activated, produce a fixed change in reporter gene DNA, and thus relate ligand binding to an enzyme activity that is readily and precisely measurable (23). Furthermore, the transient event of ligand binding becomes converted by the recombinase activity of the FLP-LBD into fixed, heritable changes in DNA. Since the human estrogen receptor is a ligand-dependent transcription factor in *S. cerevisiae* (29), we reasoned that FLP-estrogen receptor LBDs (FLP-EBDs) would also be ligand-responsive in yeast. Here we examine the relationship between ligand binding and the enzyme activity of FLP-EBD fusion proteins. Ligand titration experiments show that ligand induced recombination reflects apparent ligand binding. Furthermore, all estrogens tested, either hormones or type 1 or type 2 antihormones (type 1 are partial agonists, e.g. 4-hydroxytamoxifen and type 2 are pure antagonists, e.g. ICI 182,780) induce recombination.

We also show that the amount of estrogen receptor D domain included in the fusion protein between the FLP and EBD domains influences the activity of the recombinase. If the D domain is omitted, antihormones but not hormones, are unable to activate the fusion recombinase. The ability to distinguish between hormones and antihormones in yeast is extremely important in understanding how ligands activate the estrogen receptor. The objective of this effort is to exploit yeast-based genetic screens of mutated EBD clone banks to find mutations which specifically alter ligand binding, and hence recombination. By use of different ligands and various

concentrations, we will attempt to map specific amino acid changes to specific side-groups of ligands, thereby defining components of the binding pocket and making rational ligand design more predictable. Combining the EBD fusion point dependence for antihormone action with numerous random mutations of the estrogen binding domain has produced many EBDs which show altered or reversed activation by hormones and antihormones.

Objectives

The research being pursued involves defining the specific interactions between estrogen ligands and the estrogen binding domain to ascertain a better understanding of ligand binding and function, especially concerning hormones and antihormones. The fusion point dependence for antihormone action in our FLP recombinase/ estrogen binding domain (FLP-EBD) system allows a mechanism to study therapeutically important antihormones, such as tamoxifen and raloxifene, and why/how they act as antihormones. This strategy separates the interdigitated functions present in the estrogen binding domain and should lead to a more precise functional characterization of the domain as well as further the potential for rational drug design.

BODY

This report covers the grants, DAMD17-94-J-4103 (Sept 95-Aug 96); DAMD17-94-J-4249 (Sept 95-Aug 96). The work to date has involved characterizing FLP recombinase-estrogen receptor hormone binding domain (FLP-EBD) fusion proteins in yeast and screening mutated ligand binding domains for altered ligand interaction. Having continued to develop and improve the basic strategy, we have applied it to (a) continued mutagenic library screening, the results from such screens are presented below, and (b) ligand induced changes in yeast colony color as a simple way to differentiate estrogen hormones and antihormones. We will also continue to include work with the therapeutically important antihormones, tamoxifen and raloxifene.

Results:

Strategy for expression and screening of ligand induced FLP recombination.

The LBD of the human estrogen receptor (EBD) includes amino acids 303 to 534, as revealed by deletion analysis (20,22) and 308 to 546 by sequence alignment (51). To regulate the FLP recombinase in yeast, the human ER hormone binding domain (domains D, E, and F; aa 251-595) was fused to the C-terminus of the entire coding sequence (423 aa) of FLP recombinase. The fusion gene was cloned under the control of the GAL10 galactose promoter (Figure 1A). Thus transcription and expression is limited to galactose media, with virtually no expression in glucose media. The fusion gene was inserted into a derivative of pRS315 (40), a single-copy CEN plasmid with the LEU2 selectable marker.

To aid in the subcloning of mutated oligonucleotide libraries, various restriction sites have been modified or introduced into the estrogen LBD coding sequence (aa 306-595 of the human ER) without changing the amino acid sequence. Several unique sites downstream of the gene also allow the mutated oligonucleotide PCR products to be recloned easily. More specific details are presented in last year's report.

To measure FLP recombinase activity, a deletion recombination substrate was integrated at the TRP1 locus, and was confirmed to be present as a single copy by southern analysis (not shown). The recombination substrate includes the constitutive alcohol dehydrogenase (ADH1) promoter directing transcription of the URA3 gene, followed by a polyA signal to terminate RNA Pol II transcription (Figure 1B). The URA3 gene and a SUP11 ochre suppressor tRNA gene are flanked by FLP recombination targets

(FRTs). The $URA3^+$ gene can be positively selected by growth in the absence of uracil and negatively selected by addition of 5-fluoroorotic acid (5-FOA) which poisons $URA3^+$ cells. The SUP11 ochre suppressor tRNA gene between the FRTs allows recombination to be detected visually using the red/white $Ade2^+$ colony color assay (31). When present, the tRNA suppresses the ade2-1 ochre allele in the yeast strain and gives white colonies. If absent, the yeast cells visibly accumulate a red pigment when grown with sustaining levels of adenine. Following the second FRT is the coding region of the LacZ gene of *E. coli*. Expression from the LacZ gene to give β-galactosidase activity (and blue yeast colonies on X-gal plates) absolutely depends on deletion of the URA3 gene to juxtapose the ADH promoter and the LacZ gene (Fig. 1, data not shown). Hence this recombination substrate permits the detection of recombination by two different colony color assays or by Southern analysis.

Characteristics of estrogen inducible recombination.

To examine the kinetics and characteristics of recombination, we performed time course experiments. Cells were initially grown in glucose, and at time zero, the cells were resuspended in 2% galactose media containing 10^{-6} M estradiol. For precision, recombination was measured directly by Southern blotting (Figure 2), where quantification depends on a ratio of bands within one lane and is not affected by lane to lane DNA loading variations. Included were both the wild-type (wt) EBD (FLP-EBD_{wt}, with ER amino acids 251-595) as well as the single-substitution mutation G400V (FLP-EBD_{G400V}) form, which is known to have a lower affinity for ligands (44). Both these constructs include the complete D domain, using ER aa 251 as the fusion point. After 4 hours, consistent with data concerning onset of galactose inducible promoters (16), the products of recombination are apparent in the samples induced with estradiol. Recombination products are then produced linearly with time until about 8 hours (Figure 2, panel B). At 23 hours, little further recombination is evident in the galactose plus hormone samples, reflecting exhaustion of the substrate. Although FLP-EBD recombination responds to ligand, some recombination is apparent in the ligand free samples. This is particularly evident in the 23hr samples (Fig. 2, panel A).

Since fusing a protein domain onto a fully functional enzyme may alter enzyme activity, we used galactose inducibility to compare efficiencies of wt FLP and FLP-LBDs. Figure 2C shows that FLP-ER (FLP-$EBD_{251-595}$) or FLP-AR (with the androgen binding domain) are only marginally less active as

recombinases than unmodified FLP when the appropriate hormone is given. In this experiment at 7hr, unmodified FLP has recombined 61% of the substrate, FLP-ER 43% and FLP-AR 59%. This also documents that ligand regulation works well for both FLP-ER and FLP-AR.

To examine the ligand responsiveness of FLP-EBDs, we performed hormone concentration titrations with a variety of known estrogen hormones and antihormones (Figure 1C). Estradiol, hexestrol, and diethylstilbestrol are all known agonists, while ICI 182,780, 4-hydroxytamoxifen, nafoxidine, and raloxifene are antagonists, defective in at least some aspects of activating ER. Each of the titration experiments was performed with both FLP-EBD_{wt} and FLP-EBD_{G400V} fusion proteins. Based on the time course results (Figure 2), cells were harvested in the linear phase of recombination at 7 hours. The three agonists, estradiol, hexestrol, and diethylstilbestrol, show a similar relationship (Figure 3), i.e. each have an observed half maximal recombination of about 0.3 nM for the wild-type receptor fusion and about a 10 nM for the G400V form. These values are in good agreement with the known dissociation constants (Kds) for these ligands with the wt and G400V estrogen receptors (27,44). Thus the response of FLP-EBDs to these ligands is a simple reflection of ligand binding by the EBD.

In contrast, inducing recombination with several antihormones all show a much reduced relative Kd in yeast (Figure 4), compared to the known Kds for mammalian ERs (41,48,49). With FLP-EBD_{wt}, these compounds show half maximal inductions at about 300 nM and those for the G400V form are >1000 nM. Since we observe that the FLP-EBD_{G400V} form shows the expected reduced sensitivity when compared to the FLP-EBD_{wt}, a probable reason for these high values is the low permeability of the antihormones through yeast cell walls (24,52). These antihormones are significantly larger molecules than the agonists, which probably reduces their net internal concentration. Nevertheless, this data shows that all ligands tested, whether hormones or antihormones, induce both the wt and G400V FLP-EBD fusion proteins, though we continued in our mutation studies using only the wild-type EBD as a background.

Color plate assay for ligand function.

Southern analysis of ligand induced, FLP-EBD mediated recombination demonstrated that recombination reflects the concentration of particular ligand being tested (Figures 3,4). Therefore an $Ade2^{+}$ color plate assay for ligand induced recombination was developed as a way to visualize the

presence and concentration of ligands. Lawns of transformed cells with FLP-EBD$_{wt}$ or FLP-EBD$_{G400V}$ forms were screened for color formation with several ligand concentrations (Figure 5). A drop of ligand was applied to the center of a standard 9 cm petri plate and cells with recombined targets should develop a red color. Wild-type and G400V forms show the expected differences in red circle formation, based on their relative response to estrogen (Fig. 3). For example, similar sized red circles (about 4 cm diameter) were observed when FLP-EBD$_{wt}$ was exposed to 0.1 nmols and FLP-EBD$_{G400V}$ to 100 times more (10 nmol). The results confirm that this "circle" test reflects amounts of ligand applied as well as relative affinities. This plate assay has been the core of our ability to find and confirm mutations from libraries which show an altered ligand response relative to the wild-type EBD sequence (Table I).

Fusion point of the estrogen LBD differentially affects hormone/antihormone action.

All previous applications of estrogen receptor EBDs to regulate other fusion proteins have used aa 282 of the EBD as the fusion point (33, and references therein). This includes about half of the D domain (aa 263-305). The D domain is thought to be a flexible, unstructured hinge region between the conserved C (DNA binding) and E (ligand binding) domains (8,13,21,36,39,47). Previous work suggested that the spacing between the LBD and the regulated protein may influence the regulatory potential by the LBD (34). Since recombination presents a more precise way to assay for LBD functions than available to previous LBD fusion proteins, we compared FLP-EBDs that do or do not include the D domain (fusion at aa 251 vs. aa 304, Figure 6). Coupled with this fusion point variation, we tested both the wt and G400V forms of the EBD, as well as responses to estradiol or 4-hydroxytamoxifen (Figure 7). Removing the D domain (304) does indeed reduce the level of background observable in the absence of ligand; however the overall level of recombination mediated by the 304 construct was reduced, as was the apparent affinity for estradiol by about 10-fold (Figure 7A). Unexpectedly, we observed that the 304 constructs were essentially unactivatable by the antihormone 4-hydroxytamoxifen (Figure 7B), even at very high concentrations for 24h (Figure 8, and data not shown).

To more fully address the influence of the D domain on fusion protein repression and ligand specific inducibility, we made a more extensive comparison of hormones and antihormones with the 251 vs. the 304 fusion

constructs (Figure 8A). The deletion of the D domain has a profound effect on the fusion recombinases. Very high concentrations (10^{-5} M) of the antihormones are still unable to activate recombination of the 304 forms, whereas the 251 forms activate well under identical conditions. We tested this surprising observation further by constructing a FLP-EBD that contained part of the D domain (fusion at aa 286). A comparison of FLP-EBDs that include all (251), part (286) or none (304) of the D domain (Figure 8B) shows that the 286 construct has an intermediate phenotype. The ligand responsiveness of the 286 construct is reduced when compared to the 251 construct, however less so for estradiol than the antihormones.

Library generation by codon substitution mutagenesis (CSM)

CSM is the best method available for saturation mutagenesis of a region (10). It is an oligonucleotide based method, achieved by mixing small-scale synthesis of three random nucleotides with a large-scale synthesis containing the wild-type codon. This is repeated for each codon to be mutated and consequently involves intensive use of two oligonucleotide synthesizing machines. We have initiated this technique to be established by the oligo service here at EMBL. The initial mutation libraries focused on a region (aa 506-532) previously implicated by random single mutation data as very likely to be important for estrogen ligand specificity. We have since generated a total of eight libraries (see Figure 9) covering many of the predicted regions of importance for ligand interaction or specificity, as judged by the existing x-ray crystal structures of the retinoid receptors, RAR, RXR, and TR (8,36,47). A major improvement over the strategy outlined in the original proposal is to PCR amplify the synthesized oligo library and clone to unique sites of choice (see last year's report). This allows two advantages: (i) the inherently low chemical yields of long, mutated oligos are circumvented by PCR amplification, and (ii) subsequent cloning relies on only one unique restriction site in each region, which we have set up throughout the LBD.

Screening assay for altered specificity of ligand binding

After generating a library of mutagenized FLP-EBD constructs, we perform a screening assay as outlined in Figure 10. After plating thousands of independent yeast transformants from a mutagenesis library, we pool the colonies and grow a culture in galactose to induce transcription of the recombinase fusion gene. The cells are grown in the presence of a first

ligand and selected for non-recombination, i.e. fusion proteins which are not induced by the first ligand. The cultures are collected, washed and grown in galactose with a second ligand for which we screen for recombination, using the plate assay outlined above. Hence we enrich for plasmids containing mutations that are unreactive with a first ligand, yet still retain binding to a second ligand which results in recombination. These plasmids are retested in the parent strain to confirm differential inducibility, as compared to the wild-type sequence, by a set of ligands. Screening of the 8 libraries outlined in Figure 9 is ongoing, and results thus far are summarized in Table I.

Discussion:

FLP-EBDs are induced by ligands in a concentration dependent manner.

Hormones for the estrogen receptor show similar affinities for FLP-EBD_{wt} in yeast as they do for the full length receptor expressed in mammalian systems. The FLP-EBD_{G400V} shows about 30-100X lower affinity when compared to FLP-EBD_{wt} (Figure 3), as expected from the reduced agonist affinities to full-length G400V receptor (44). We observe that agonist inductions of these FLP-EBDs therefore accurately reflect ER binding affinities and conclude that all parts of ER required for full agonist binding affinity are present. In addition, FLP-AR fusion proteins containing equivalent D+E+F domains of the human androgen receptor are also activated with wild-type affinities by androgens (Figure 2C; not shown).

Antagonists, however, require higher concentrations to activate the FLP-EBDs in yeast relative to ER in mammalian cells, probably because of reduced permeability into yeast cells, as has been described (24,52). In those studies, yeast extracts containing ER had normal ligand affinities, implying permeability as the reason for low antagonist activity (18,24,52). Consistent with this explanation, FLP-EBD_{G400V} needs much higher concentrations of antagonists than FLP-EBD_{wt} (Figure 4), as expected from its reduced affinity for ligands.

Although FLP-EBDs including all of the D domain display agonist inductions equivalent to full-length ER affinities, this is not the case for constructs that omit the D domain. FLP-EBD_{304}, which contains none of the D domain, displays reduced inducibility by estradiol (Figure 7) and virtually no inducibility by antagonists (Figures 7,8). The recent crystal structures of three nuclear receptor LBDs (8,36,47) strengthens the evidence that the D domain does not play any direct role in ligand binding. Therefore either the D domain plays an indirect role or the close juxtaposition of FLP

recombinase to the EBD interferes with recombinase activation by ligand. A combination of both explanations is also possible.

Fusing the estrogen LBD closer to FLP selectively blocks activation by antihormones.

Using transcription assays in yeast, classical ER antihormones in mammalian cells behave as weak agonists and do not antagonize estradiol activation, even at very high (5 μM- 200 μM) concentrations (6,24,29,45,50,52). The partial agonist transcriptional activity of 4-hydroxytamoxifen in yeast and mammalian cells results from the action of AF-1 when the ER is bound to an ERE, as AF-2 can be deleted (6,46). The properties that determine whether a ligand acts as a hormone or an antihormone are unclear, though it is almost certain that different conformations of the LBD and the AF-2 transcriptional surface that result from ligand binding primarily determines agonists vs. antagonists (1,2,5,26). ER antihormones are thought to interrupt the presentation of the hormone-dependent AF-2 activation surface, resulting from an altered conformation after antihormone binding. In the FLP-EBD recombinase assay described here, all ligands, whether agonists or antagonists, induce recombination by the FLP-$EBD_{251-595}$ forms in a concentration dependent manner that appears to be a simple reflection of internal ligand concentration. The assay requires only that ligand binds and causes a conformational change to derepress the FLP-EBD. Subsequent presentation of AF-2 or DNA binding to an ERE is not measured, however the EBD must adopt a conformation that does not interfere with the FLP recombination mechanism, which involves four protein monomers (9). Antihormones are much larger than hormones and probably interrupt the relatively compact LBD structure that would form around bound hormones (36,47). The D domain is thought to act as a flexible linker between the C domain zinc fingers and the LBD of natural hormone receptors, or between FLP and the LBD in our assay. However when the D domain is removed and the LBD is closer, as in the FLP-$EBD_{304-595}$ form, hormone induction is impaired and antihormone induction is prevented. We reason that the antihormone-induced conformation of the EBD interferes with the FLP reaction, possibly by not allowing the FLP tetramers to align as an intermediate to recombination. The intermediate phenotype of the 286 fusion, which shows that the antagonist sensitivity to shortened D domains is not an all-or-none phenomenon, is compatible with this model. Since AF-2 activity is not transcriptionally measurable in yeast, this presents a first yeast assay for the discrimination between hormones

and antihormones. For example, *a compound which induces recombination with the 251 form but not the 304 form likely has antihormone properties and can be tested in full-size ER assays for confirmation.* Taken together, this presents a functional assay to probe the difference between hormone and antihormone action on the estrogen receptor in yeast.

LBD-fusion proteins regulated by a proximal Hsp90 complex.

Current models to explain how LBDs regulate the proteins to which they are fused invoke a primary role for the Hsp90 complex (33). The Hsp90 complex is ubiquitous and abundant, and possesses chaperonin activity (35). Further recent evidence that the steroid receptor LBDs are associated with this complex in the unliganded state has come from genetic experiments with yeast (7,17,30). Fusion of an LBD onto a heterologous protein is believed to direct the fusion protein to associate with the Hsp90 complex. Binding of agonists promotes LBD release from the complex, thus derepressing the fusion protein functions. Whether all antagonists serve to release LBDs from the Hsp90 complex to the same extent remains unclear. However, we observe that all ligands, regardless of whether they are agonists or antagonists, induce recombination by FLP-EBD251-595. We therefore conclude that all of these ligands, which include partial antagonists [4-hydroxytamoxifen, (6)] and complete antagonists [ICI 182,780, (12,48)] induce release from the Hsp90 complex.

It was shown before that the distance between the LBD and the regulated protein was important to complete ligand regulation. When the distance was artificially increased between E1A protein and a glucocorticoid receptor (GR) LBD, regulation was lost and background activity without hormone was significant (34). Likewise we observe that the FLP-EBD251-595 forms are partially active without ligand at later times (Figure 2A), but the shorter FLP-EBD304-595 forms show no background whatsoever (Figure 7, 8; unpublished data), presumably because the Hsp90 complex is closer to the recombinase.

Materials and Methods

Strains and chemicals.

The yeast strain used for these experiments (MAT **a**, leu2-3,112, his3-11,15, ura3-52, trp1-1::(TRP1,URA3,SUP11), ade2-1^{ochre}, can1-100) was derived from RS453 (R. Serrano, Valencia, Spain) by integrating the target of recombination (Fig. 1B) at the *trp1* locus (PmlI site). Transformation of yeast by the LiAc method was performed as described (3). Transformed yeasts were grown and maintained with selection for leucine and tryptophan in glucose or galactose supplemented synthetic media from BIO 101, Inc. The hormones and antihormones were purchased from Sigma, except 4-hydroxytamoxifen (Research Biochemicals International), mibolerone (New England Nuclear), and ICI 182,780 (a gift from Dr. A. Wakeling, Zeneca Pharmaceuticals).

Southern assays, ligand titration experiments, and quantification.

Transformed yeast, containing the GAL10 promoter, FLP-EBD gene on pRS315 (40), were grown in synthetic glucose medium lacking leucine and tryptophan to OD_{600} of about 1.5. Equal volumes of cultures were collected and resuspended in medium containing 2% galactose with or without hormone. Cells were collected at times noted and DNA was prepared by standard procedures using a zymolyase 20T (ICN) incubation, SDS lysis, followed by potassium acetate precipitation as described (3). About 10 μg of DNA per lane was digested with PstI and loaded on 0.7% gels in 1x TAE buffer. Gels were treated with 0.25M HCl for 10 min, 0.4M NaOH for 2x 30 min, 20x SSC for 30 min, and then blotted to Qiagen nylon plus filters with 20x SSC. After baking the filter at 80^{o} C for 2 hr, they were probed at 72^{o} C with a riboprobe made from the 1.2 kb ScaI-BsiWI fragment of the *E. coli* LacZ gene in a buffer containing 250 mM Na^{+} phosphate pH 7.2, 7% SDS, and 1 mM EDTA. Washes were performed in 25 mM Na^{+} phosphate pH 7.2, 1% SDS and 1 mM EDTA at 72^{o}C. Radioactive bands on the filters were quantified using the Phosphorimager system by Molecular Dynamics, Inc. Observed recombination was calculated as the ratio within a lane [counts in the recombined band/ (counts in recombined + unrecombined bands)], and is therefore not affected by minor variations in the amount of DNA loaded.

Plate assay.

Yeast containing the integrated SUP11 recombination substrate and either the wt or G400V FLP-EBD fusion proteins were plated at high density on synthetic galactose plates lacking leucine and tryptophan. A 1 μl drop of ethanol containing ligand (or not) was placed at the center of the plate. The plates were grown at 30^{o} C for 4 days to maximize red color formation.

CONCLUSIONS

The components of estrogen ligand-inducible recombination mediated by FLP-EBD fusion proteins have been established as an assay in yeast. The properties of the assay show that all ligands are able to activate FLP-EBD fusion proteins when fused from amino acid (aa) 251 of ER. Fusions from aa 304 report only hormone binding, whereas antihormone binding probably forms an EBD conformation which blocks recombination by a steric mechanism. This presents a simple assay to predict hormone vs. antihormone activity of a compound in yeast, as well as a way to screen for functional interactions with amino acids defining hormones vs. antihormones. We have used codon substitution mutagenesis (CSM) to generate 8 different mutagenized EBD libraries. Estrogen induced changes in yeast colony color has been used as a simple method to detect ligand binding, and to measure its relative hormone and antihormone character.

In the coming year we will:

(a) construct and screen further CSM libraries of EBD mutations and continue analysis of those already in progress. These libraries will be screened for mutations that alter (i) responsiveness to known estrogen hormones and antihormones (Fig 1); (ii) responsiveness to ligands that are very poor estrogens; (iii) the repressive functions of the EBD, as assayed by either their constitutive activity or failure to respond to authentic estrogens.

(b) further develop the potential of the assay as a method to detect and measure estrogenic activities. This will include work addressing estrogens from environmental samples of potentially hazardous manufacturing waste.

(c) alter the N and C termini of the EBD in FLP-EBD fusion proteins in order to define the functional boundaries of the EBD with regard to its repressive properties.

(d) perform direct ligand binding assays to measure ligand affinity changes with many of the mutations already generated.

(e) reclone and test in the natural full-sized estrogen receptor (ER) the effects of particular mutations on transcription in reporter assays.

These studies address two important questions. What are the amino acid determinants of ligand binding specificity in the estrogen receptor? Can the FLP-EBD fusion proteins define precisely the functional components of hormone versus antihormone action? These will both be decisive in understanding how tamoxifen and raloxifene bring about their positive effects in present endocrine therapies, and in developing better therapeutic agents for the future.

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Figure Legends

Figure 1. (A) Diagram of the FLP-EBD expression plasmid displaying its functional elements. The gene for the FLP-ER LBD fusion protein is driven by a galactose promoter on a plasmid in yeast. (B) Diagram of the FLP recombination deletion strategy. Before recombination, the constitutively active ADH1 promoter expresses the URA3 selectable marker which lies between directly repeated FLP recombination targets (FRTs, shown as triangles). The polyadenylation signals (pA) after URA3 prevent LacZ expression. Also lying between the recombination targets is a SUP11 gene which is transcribed in the opposite direction, as depicted by the short arrow. Downstream of the second recombination target is the LacZ coding region. After recombination, the URA3/SUP11 region is excised and the LacZ gene juxtaposed to the ADH1 promoter. Recombination mediated alterations in cellular phenotype are displayed at the right of the diagram. Note that expression of the endogenous Ade2+ gene relies on SUP11 expression. The diagram also outlines the Southern strategy employed. A 5.6 kb fragment is reduced by recombination to a 4 kb fragment when a probe from the LacZ gene is used. (C) Structures of the ligands tested with the FLP-EBD. The hormones are shown above the line, antihormones below.

Figure 2. (A) A time course of induction of recombination after the addition of yeast cells to galactose media containing 10^{-6} M estradiol (+) or not (-). The onset of recombinase activity reflects the 2-3 hour lag for galactose-inducible promoters. Recombination is approximately linear from 4 hours to 8 hours. (B) The Southern blots were quantified by PhosporImager analysis, and observed recombination, as determined by the ratio [counts in the recombined band/ (counts in recombined + unrecombined bands)], was calculated for each hormone treated sample and plotted. (C) The recombinase activities of the wild-type FLP protein and FLP-ER ($FLP\text{-}EBD_{251-595}$) and FLP-AR ("DEF" domains of the human androgen receptor) fusion proteins is compared. Samples were cultured for 7 hours in glucose (1st lane) or galactose without (-) or with (+) ligands. For FLP-ER, 10^{-6} M estradiol was used, for FLP-AR, 10^{-6} M mibolerone was used.

Figure 3. Titration curves of estrogen agonists show that induction of FLP-EBD in yeast accurately reflect the natural mammalian binding affinities. Both wild-type (wt) and G400V forms of the FLP-EBD_{251} were tested against titrations of various ligands. Panel A shows the southern blot for estradiol, as an example. Ligand was added (+), or not (-), to the galactose medium to give final molar concentrations as indicated. Cells maintained in glucose medium are indicated as gl. Cells were harvested at 7 hours. Panel B shows the titration data after phosphorimager quantification, for the three estrogen agonists. The G400V mutated estrogen binding domain shows 30-100X lower affinity for each of the hormones (compare squares (wt) versus the circles (V400).

Figure 4. Titration curves for several estrogen antagonists. Panel A shows the southern blot data for ICI 182,780, as an example. Panel B shows plots of phosphorimaging data for all four of the antihormones tested. In all cases, inducing ligand concentrations for the FLP-EBD_{wt} and G400V proteins are lower than for the corresponding full-length estrogen receptor in mammalian cells.

Figure 5. FLP-EBD recombination in a color plate assay in yeast reflects affinity and the concentration of a hormone placed on a lawn of unrecombined yeast cells. Yeast containing the SUP11 recombination substrate (Fig. 1) and either the wt (FLP-$EBD_{251-595}$)or G400V FLP-EBD fusion proteins were plated at high density onto low adenine medium in a standard 9 cm petri dish. An adenine marker in yeast gives a color phenotype (red) if the target gene has been deleted by the recombinase. A 1 μl drop of ethanol containing none, 10 nmol or 100 pmol of estradiol was placed at the center of the plated yeast lawn and then grown for several days. The affinity for estradiol by the wt EBD is greater than the V400 EBD, hence it shows a larger red circle (the size reflects 30-100X higher affinity by the wt EBD). If only 1/100 as much hormone is added, both circles of red colonies are correspondingly smaller. The reaction with the wt form gives about the same size red circle (about 4 cm diameter) as the V400 form when 1/100 as much hormone is added, correlating with the affinity difference for estradiol.

Figure 6. (A) Schematic diagram of the ER LBD. (B) The protein sequence of the C, D, E, and F domains. The fusion points used for the FLP chimeras are signalled by the arrows at 251, 286, or 304. The structural elements of the hormone binding domain, deduced from sequence alignments and the known RAR structure (36,51), are mapped onto the sequence of the human ER. Boxes outline α -helices (H1 to H12) and arrows mark β-sheets (S1, S2).

Figure 7. The fusion point between FLP and the EBD determines its activity in yeast. In combination without (wt) or with the G400V mutation, FLP-EBDs containing the D domain ("251") or not ("304") were compared. Panel A shows estradiol titrations, panel B shows 4-hydroxytamoxifen titrations. The phosphorimager quantification of recombination for each panel is plotted below the southern blots.

Figure 8. The D domain selectively alters ligand responsiveness. Panel A shows that the deletion of the D domain ("304") renders the fusion recombinase insensitive to any of the antihormones tested. Parallel inductions with the $FLP\text{-}EBD_{wt}$ ("251") serve as controls. The figure shows inductions by hormones (E-estrogen, D-diethylstilbestrol and H-hexestrol) at 10^{-7} M and antihormones (Z-OHT- 4-hydroxytamoxifen, RAL-raloxifene and ICI 182,780) at the concentrations indicated. Panel B shows FLP-EBD ligand inducibilities of an intermediate fusion point, at aa 286, compared to parallel inductions with $FLP\text{-}EBD_{wt}$ ("251") and D domain deletion ("304") proteins.

Figure 9. The protein sequence of the C, D, E, and F domains of the human estrogen receptor (see Fig. 6). The regions of independent codon substitution mutagenesis libraries are outlined by large boxes and are labelled A through H. The fusion points used for the FLP chimeras are signalled by the arrows at 251, 286, or 304. The structural elements of the hormone binding domain, deduced from sequence alignments and the known RAR structure (36,51), are mapped onto the sequence of the human ER. Boxes outline α -helices (H1 to H12) and arrows mark β-sheets (S1, S2).

Figure 10. Screening procedure for isolation of mutations which show altered ligand reactivities, relative to the wild-type EBD. A pool of thousands of independent yeast transformants is grown in a galactose culture to induce transcription of the recombinase fusion gene. A first ligand is included (e.g. estradiol) with selection against recombination to enrich for non-ligand binding FLP-EBDs. The example presented here must maintain the URA3 gene between recombination targets. The cells are collected and grown in the presence of a second ligand without selection, and then screened on plates for the loss of the insert DNA (hence red colony, see Fig. 1). The FLP-EBD clone is recovered and tested, versus wild-type, as a pure cultured lawn of yeast in the plate assay with a panel of ligands. E2- estradiol, OHT- 4-hydroxytamoxifen, Ral- raloxifene, DES- diethylstilbestrol, Tam- tamoxifen, ICI- ICI 182,780.

Table I

Colonies which show altered ligand specificity with amino acid changes in the human estrogen binding domain.

We used the wild-type FLP-EBD304-595 form as the parent where:
-antihormones are selectively unable to activate the recombinase fusion
-background recombination in the absence of ligand is virtually zero

(1). FLP-EBD304-595 with wild-type EBD:

Hormones activate; antihormones do not

E2 ≥ DES = Hex >>> ZOHT > Tam > Ral > ICI

E2-estradiol, ZOHT-4-hydroxytamoxifen, Ral-raloxifene, DES-diethylstilbestrol, Tam- tamoxifen, ICI- ICI 182,780

MUTATIONS DEMONSTRATING:

(2). Reversed phenotype: antihormone active, hormone inactive

175 isolates

25 separate sequences

(10 very active; 11 moderately active; 4 very weak)

(3). Relaxed Phenotype:

recombinase activated by both hormones, antihormones

35 isolates

12 separate sequences

(4). Changes in one hormone activation vs. another:

23 isolates

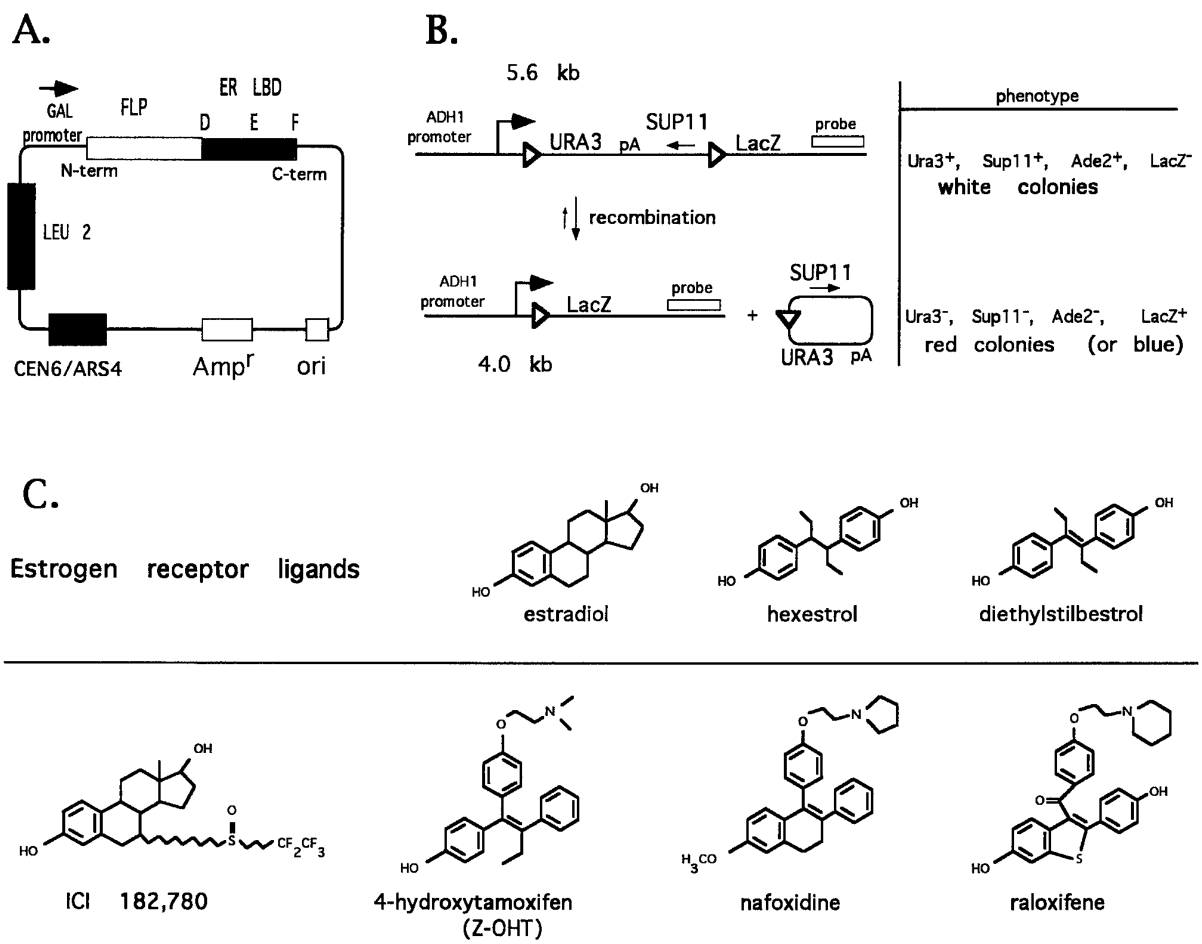
7 separate sequences

(5). Other mutations were approximately wild-type: > 5000 isolates

about 125 sequenced

(6). Mutations with no inducible recombinase activity: > 2000 isolates

about 80 sequenced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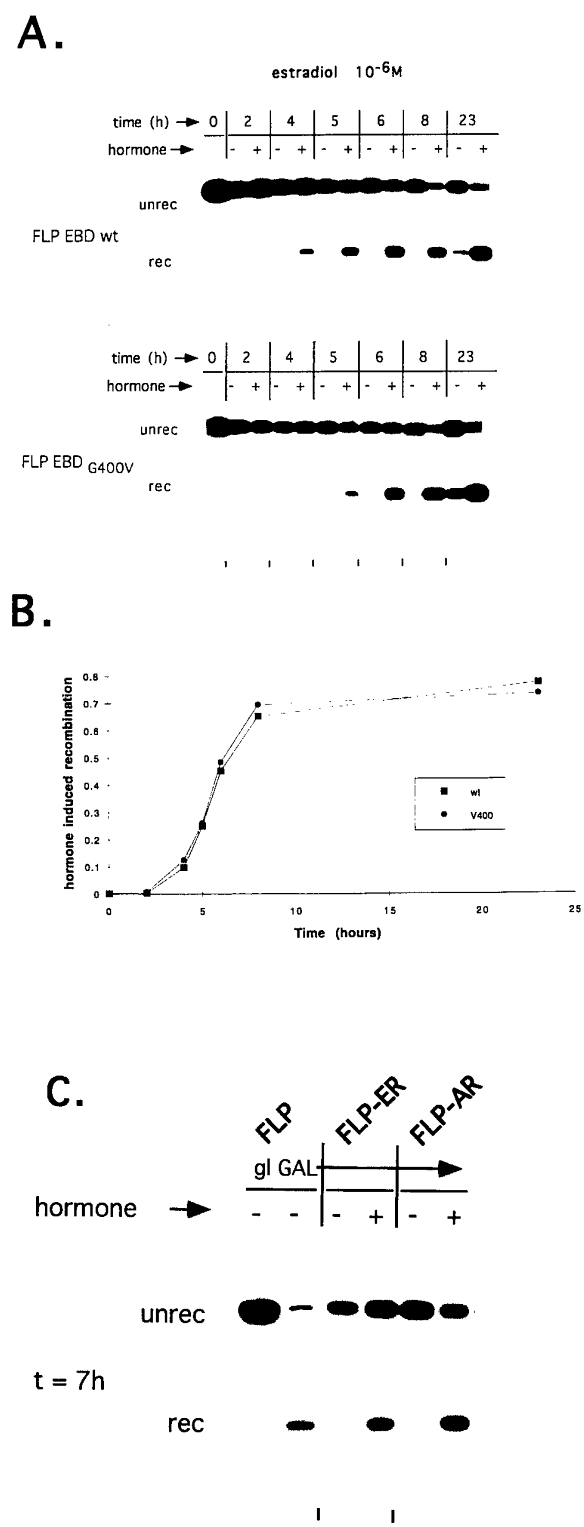
GAL promoter
FLP
ER LBD
D E F
N-term
C-term
LEU 2
CEN6/ARS4
Ampʳ
ori
B.
5.6 kb
ADH1 promoter
URA3
pA
SUP11
LacZ
probe
recombination
ADH1 promoter
LacZ
probe
SUP11
URA3 pA
4.0 kb
phenotype
Ura3+, Sup11+, Ade2+, LacZ-
white colonies
Ura3-, Sup11-, Ade2-, LacZ+
red colonies (or blue)
C.
Estrogen receptor ligands
estradiol
hexestrol
diethylstilbestrol
ICI 182,780
4-hydroxytamoxifen
(Z-OHT)
nafoxidine
raloxifene

Figure 1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unrec
FLP EBD wt
rec
time (h) 0 2 4 5 6 8 23
hormone - + - + - + - + - + - +
unrec
FLP EBD G400V
rec
B.
hormone induced recombination
Time (hours)
wt
V400
C.
FLP
FLP-ER
FLP-AR
gl GAL
hormone - - - + - +
unrec
t = 7h
rec

Figure 2

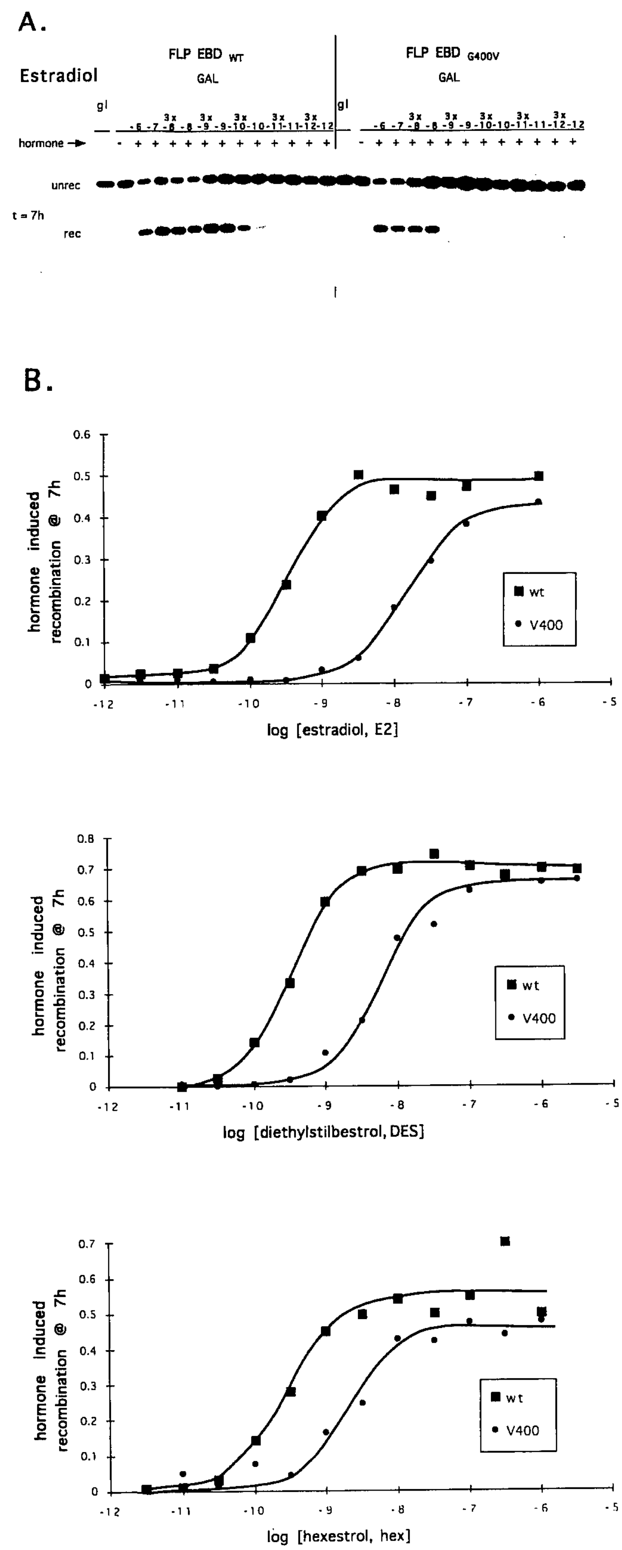
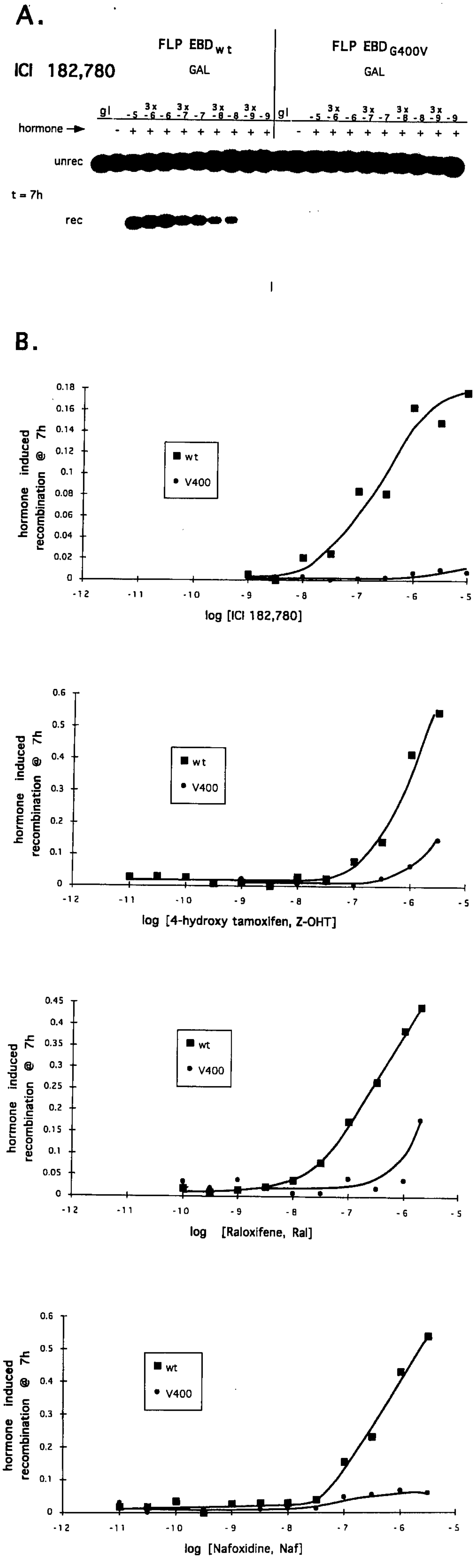

A.
FLP EBD WT
GAL
FLP EBD G400V
GAL
Estradiol
gl
hormone
unrec
t = 7h
rec
B.
hormone induced recombination @ 7h
wt
V400
log [estradiol, E2]
log [diethylstilbestrol, DES]
log [hexestrol, hex]

Figure 3


A.
ICI 182,780
FLP EBDwt
GAL
FLP EBDG400V
GAL
hormone
unrec
t = 7h
rec
B.
hormone induced recombination @ 7h
wt
V400
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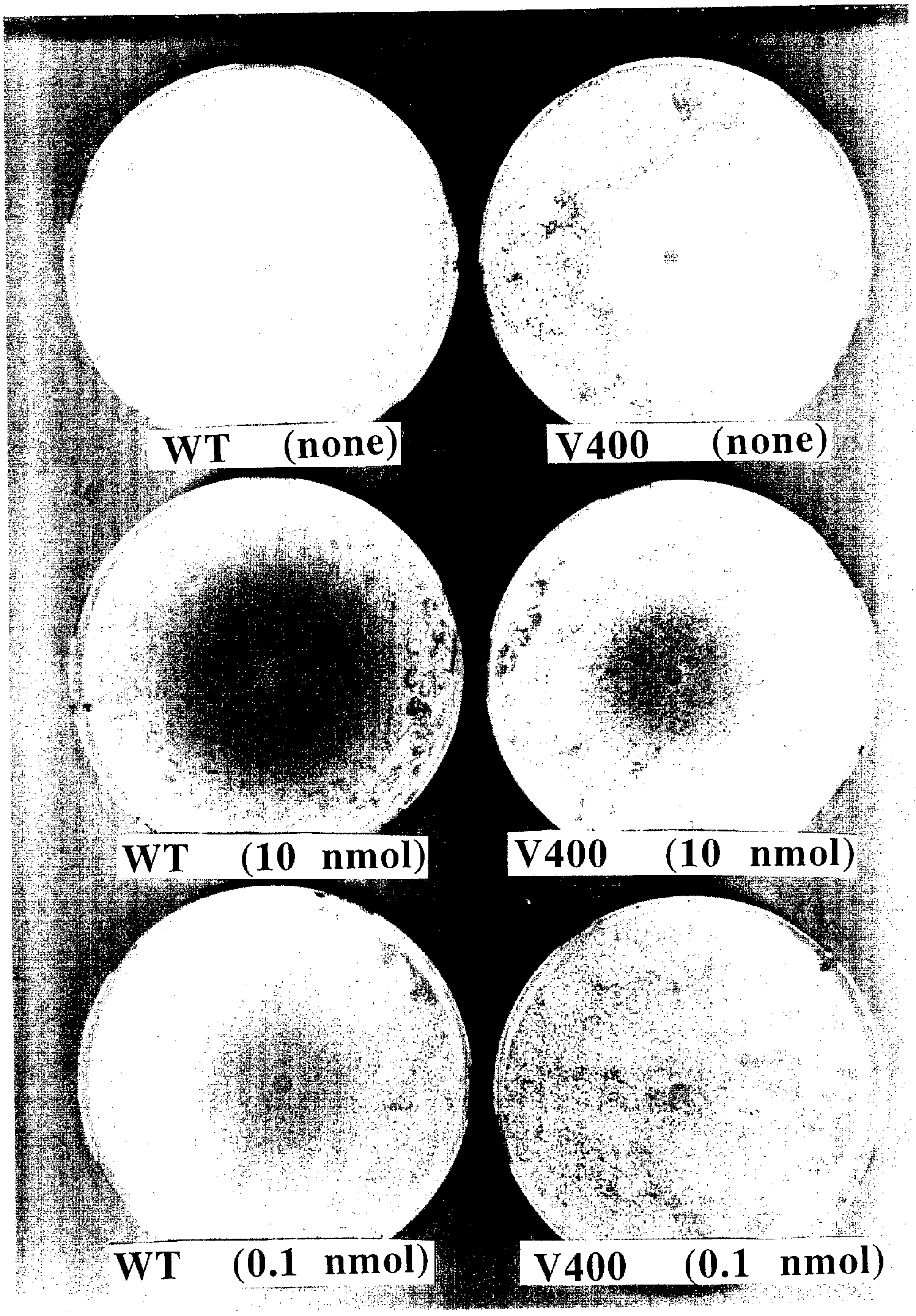
log [4-hydroxy tamoxifen, Z-OHT]
log [Raloxifene, Ral]
log [Nafoxidine, Naf]

Figure 4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V400 (none)
WT (10 nmol)
V400 (10 nmol)
WT (0.1 nmol)
V400 (0.1 nmol)

Figure 5

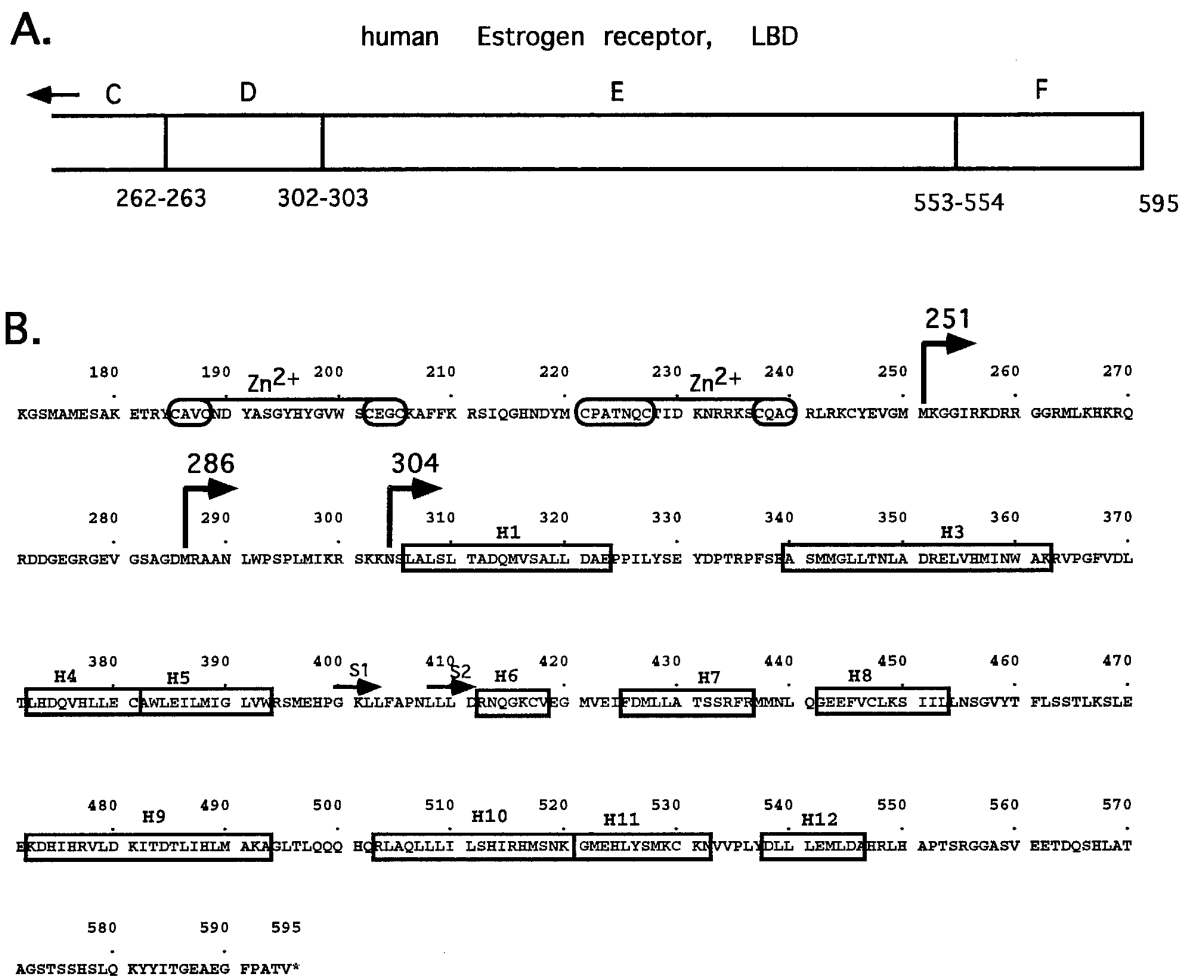
A.
human Estrogen receptor, LBD
C
D
E
F
262-263
302-303
553-554
595
B.
251
286
304
Zn2+
Zn2+
180 190 200 210 220 230 240 250 260 270
KGSMAMESAK ETRYCAVCND YASGYHYGVW SCEGCKAFFK RSIQGHNDYM CPATNQCTID KNRRKSCQAC RLRKCYEVGM MKGGIRKDRR GGRMLKHKRQ
280 290 300 310 320 330 340 350 360 370
H1
H3
RDDGEGRGEV GSAGDMRAAN LWPSPLMIKR SKKNSLALSL TADQMVSALL DAEPPILYSE YDPTRPFSEA SMMGLLTNLA DRELVHMINW AKRVPGFVDL
380 390 400 410 420 430 440 450 460 470
H4
H5
S1
S2
H6
H7
H8
TLHDQVHLLE CAWLEILMIG LVWRSMEHPG KLLFAPNLLL DRNQGKCVEG MVEIFDMLLA TSSRFRMMNL QGEEFVCLKS IILLNSGVYT FLSSTLKSLE
480 490 500 510 520 530 540 550 560 570
H9
H10
H11
H12
EKDHIHRVLD KITDTLIHLM AKAGLTLQQQ HQRLAQLLLI LSHIRHMSNK GMEHLYSMKC KNVVPLYDLL LEMLDAHRLH APTSRGGASV EETDQSHLAT
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Figure 6

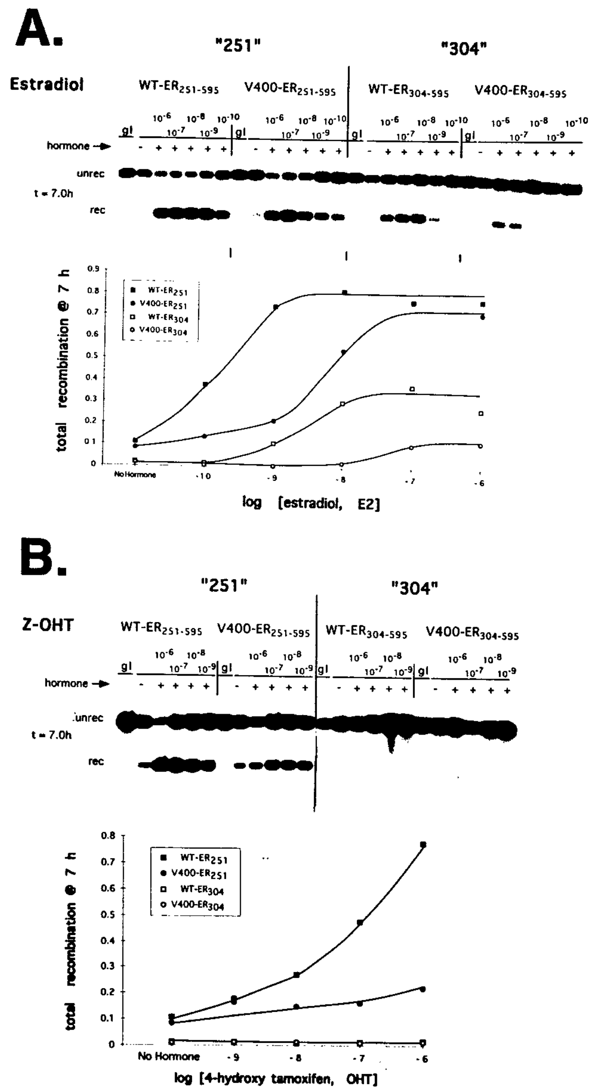


Figure 7

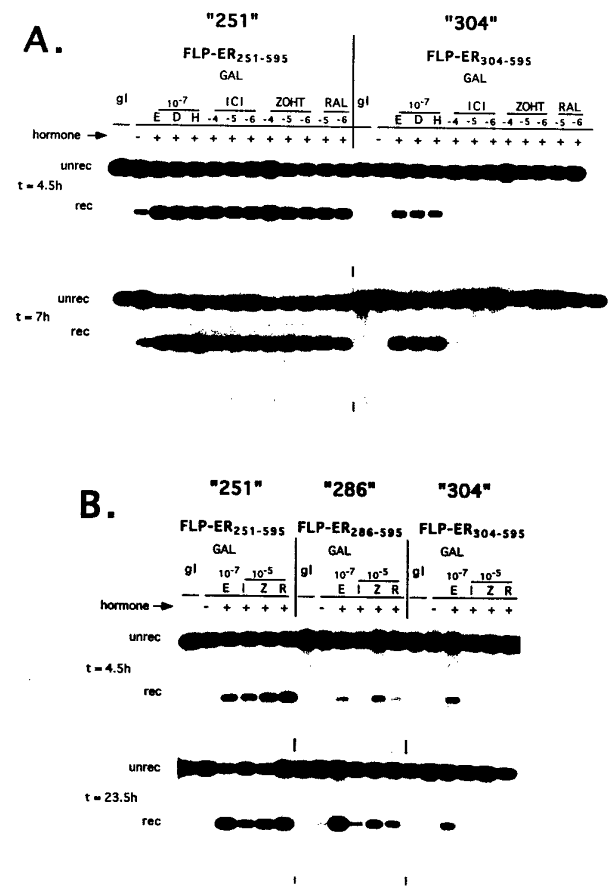
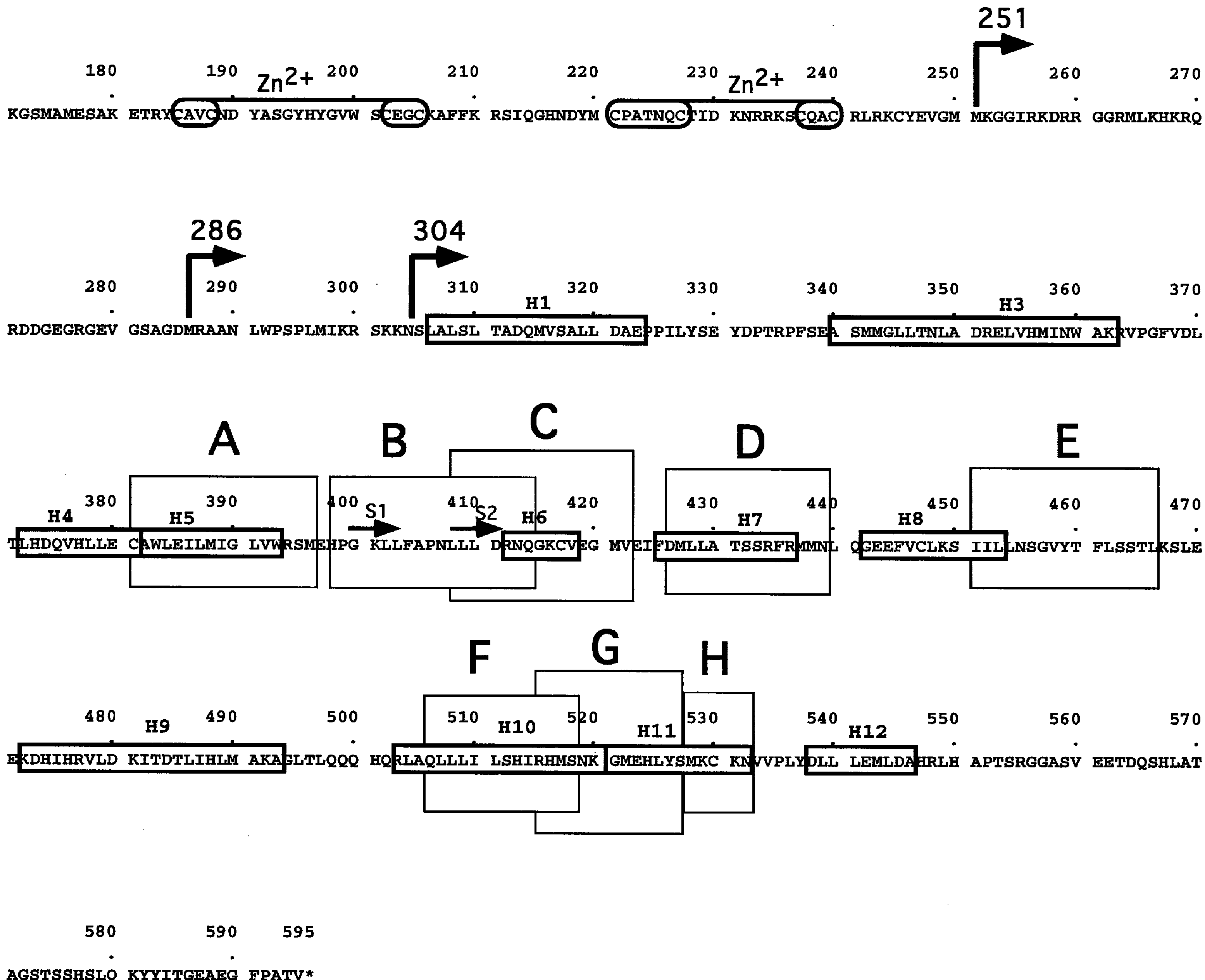


Figure 8



251
286
304
Zn2+
Zn2+
180 190 200 210 220 230 240 250 260 270
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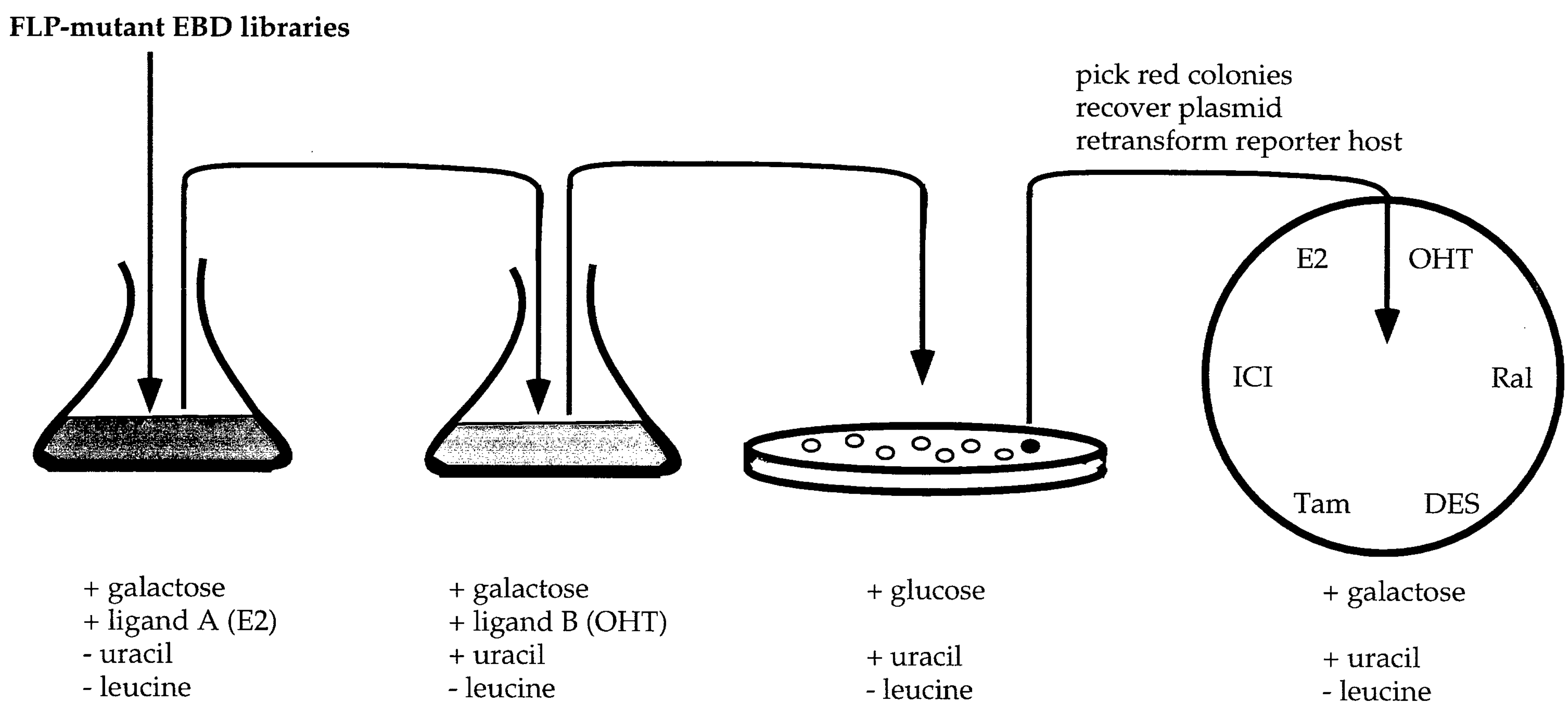
280 290 300 310 320 330 340 350 360 370
H1
H3
RDDGEGRGEV GSAGDMRAAN LWPSPLMIKR SKKNSLALSL TADQMVSALL DAEPPILYSE YDPTRPFSEA SMMGLLTNLA DRELVHMINW AKRVPGFVDL
A
B
C
D
E
380 390 400 410 420 430 440 450 460 470
H4
H5
S1
S2
H6
H7
H8
TLHDQVHLLE CAWLEILMIG LVWRSMEHPG KLLFAPNLLL DRNQGKCVEG MVEIFDMLLA TSSRFRMMNL QGEEFVCLKS IILLNSGVYT FLSSTLKSLE
F
G
H
480 490 500 510 520 530 540 550 560 570
H9
H10
H11
H12
EKDHIHRVLD KITDTLIHLM AKAGLTLQQQ HQRLAQLLLI LSHIRHMSNK GMEHLYSMKC KNVVPLYDLL LEMLDAHRLH APTSRGGASV EETDQSHLAT
580 590 595
AGSTSSHSLQ KYYITGEAEG FPATV*

Figure 9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pick red colonies
recover plasmid
retransform reporter host
E2
OHT
ICI
Ral
Tam
DES
+ galactose
+ ligand A (E2)
- uracil
- leucine
+ galactose
+ ligand B (OHT)
+ uracil
- leucine
+ glucose
+ uracil
- leucine
+ galactose
+ uracil
- leucine

Figure 10